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Mutants of the *Eco*RI endonuclease with promiscuous substrate specificity implicate residues involved in substrate recognition

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The *Eco*RI restriction endonuclease cleaves DNA molecules at the sequence GAATTC. We devised a genetic screen to isolate *Eco*RI mutants with altered or broadened substrate specificity. *In vitro*, the purified mutant enzymes cleave both the wild-type substrate and sites which differ from this by one nucleotide (*Eco*RI star sites). These mutations identify four residues involved in substrate recognition and catalysis that are different from the amino acids proposed to recognize the substrate based on the *Eco*RI - DNA co-crystal structure. In fact, these mutations suppress *Eco*RI mutants altered at some of the proposed substrate binding residues (R145, R200). We argue that these mutations permit cleavage of additional DNA sequences either by perturbing or removing direct DNA - protein interactions or by facilitating conformational changes that allosterically couple substrate binding to DNA scission.

Key words: allosteric activation, DNA binding, DNA repair, *Escherichia coli*, restriction - modification

Introduction

Many site-specific DNA binding proteins belong to one of four families, each distinguished by a different conserved DNA binding motif: the helix - turn - helix (Pabo and Sauer, 1984), the zinc finger (Miller *et al.* 1985; Evans and Hollenberg, 1988), the leucine zipper/scissors-grip (Landschulz *et al.*, 1988; Vinson *et al.*, 1989), or the helix - loop - helix (Murre *et al.*, 1989). However, other sequence-specific DNA binding proteins are not related to these families (Vershon *et al.*, 1986; McClarin *et al.*, 1986). For example, although the *Eco*RI restriction endonuclease binds and cleaves a specific DNA sequence, the enzyme is not homologous to other DNA binding proteins (Newman *et al.*, 1981; Greene *et al.*, 1981). The crystal structure of an *Eco*RI endonuclease - DNA complex revealed that each monomer of the *Eco*RI dimer steeply projects two non-contiguous α-helices into the major groove of the DNA (Frederick *et al.*, 1984; McClarin *et al.*, 1986). The ends of these helices bear amino acids which have been proposed to mediate DNA binding specificity by hydrogen bonding with the nucleotides of the recognition site (McClarin *et al.*, 1986). The *Eco*RI enzyme may employ this novel DNA recognition domain, the 'double-barrelled helix' (Vinson *et al.*, 1989), both to bind its short target sequence (three nucleotides per monomer) and to coordinate binding with catalysis.

The *Eco*RI endonuclease is a symmetrical homodimer of known sequence (Newman *et al.*, 1981) that cleaves the DNA sequence GAATTC between the G and A of both strands (Hedgpeth *et al.*, 1972). N^6-methylation of the central adenine residues by the *Eco*RI methylase protects the site from cleavage by the endonuclease. *In vitro* studies revealed that the *Eco*RI endonuclease initially binds DNA non-specifically and then diffuses in a one-dimensional search to locate and cleave its recognition site with exceedingly high fidelity (Jack *et al.*, 1982; Terry *et al.*, 1985; Halford and Johnson, 1980; Thielking *et al.*, 1990). Certain buffer conditions (low salt, basic pH, glycerol) or co-factors (Mn^{2+} instead of Mg^{2+}) favor scission at additional DNA sequences that usually differ by one nucleotide from the canonical substrate, a phenomenon known as *Eco*RI* (* = star) activity (Polisky *et al.*, 1975; Hsu and Berg, 1978; Malyguine *et al.*, 1980; Woodbury *et al.*, 1980a; Gardner *et al.*, 1982; Rosenberg and Greene, 1982; see also Thielking *et al.*, 1990). Based on crystallographic (McClarin *et al.*, 1986), kinetic (Terry *et al.*, 1987), thermodynamic (Ha *et al.*, 1989), and genetic evidence (Jen-Jacobson *et al.*, 1983, 1986; King *et al.*, 1989; Wright *et al.*, 1989), it has been proposed that sequence-specific DNA interactions induce conformational changes in the *Eco*RI enzyme that allosterically couple DNA binding and scission.

To identify amino acids involved in DNA recognition, we developed a genetic screen to isolate *Eco*RI endonuclease mutants with altered or disrupted substrate specificity. As a first step, we mutated those residues of *Eco*RI implicated in substrate recognition by the co-crystal structure (McClarin *et al.*, 1986). Surprisingly, none of these mutations altered the specificity of the enzyme (Heitman, 1989; Heitman *et al.*, 1989c; Heitman and Model, 1990), as has also been observed by others for conservative (Wolfes *et al.*, 1986; Alves *et al.*, 1989) and non-conservative mutations (Needels *et al.*, 1989). These studies all support the conclusion that the hydrogen bond network proposed from the crystal structure is not sufficient to explain *Eco*RI substrate specificity.

In our second step to identify residues of *Eco*RI that recognize the substrate, we devised a genetic screen that did not depend upon prior knowledge of the protein structure. In this approach we isolated *Eco*RI mutants which damage the DNA of their host cell despite the presence of the *Eco*RI methylase. These mutant enzymes have been purified and their DNA cleavage specificities determined *in vitro*. The responsible amino acid substitutions identify four residues that are involved in substrate recognition and cleavage. In addition, these mutations act as genetic suppressors when recombined with a set of previously isolated mutations in the *Eco*RI substrate binding pocket, lending support to the *Eco*RI allosteric activation model in which conformational changes couple DNA binding and cleavage (McClarin *et al.*, 1986; Terry *et al.*, 1987). Lastly, we suggest that the genetic

screen described here should be widely applicable to the study of other enzymes that interact with DNA such as recombinases, topoisomerases, and DNA repair enzymes.

Results

Isolation of EcoRI endonuclease mutants that evade the EcoRI methylase to damage the chromosome of the host cell

Our strategy to isolate *Eco*RI mutants altered or reduced in substrate specificity is based on our finding that DNA scission *in vivo* induces the *E.coli* SOS DNA repair response and increases β-galactosidase expression in strains that carry the lactose operon fused to a DNA damage inducible promoter (Kenyon and Walker, 1980; Heitman and Model, 1987, 1990; Heitman *et al.*, 1989a). Here we have isolated *Eco*RI endonuclease mutants which damage the DNA of their host cells and induce the SOS response, despite the presence of the *Eco*RI methylase. Because such mutants might be lethal, they were isolated from a temperature-sensitive (TS) *Eco*RI allele (TS6=R56Q) to allow conditional expression (Heitman *et al.*, 1989a; this TS mutation, R56Q, was also recovered by others in an independent screen in which the TS phenotype went unrecognized: Yanofsky *et al.*, 1987).

A plasmid bearing the TS6 *Eco*RI endonuclease allele was mutagenized *in vivo* with nitrosoguanidine or by growth in a *mutD* mutator strain (see Materials and methods). Purified plasmid DNA from independently mutagenized cultures was introduced into the SOS::*lacZ* fusion strain JH137 carrying a plasmid expressing the *Eco*RI methylase (pJC1). The resulting transformants were plated on X-gal indicator medium and screened for blue (SOS induced) colonies at temperatures partially (37°C, 34°C) and fully (30°C) permissive for endonuclease activity of the TS *Eco*RI allele. About 30 mutants were obtained by this means at a frequency of ~0.2%. One additional allele (B208) was found by screening ~20 000 replica-plated colonies for ones that grew poorly or were unable to grow at 30°C.

Mutations which clearly induced the SOS response in a TS fashion were all plasmid-linked and mapped within specific regions of the endonuclease gene, each defined by a deletion spanning part of the gene (see Materials and methods). Amongst these mutants, four different amino acid substitutions were identified by sequencing the appropriate portion of the gene (Figure 1). These included two independent isolates of H114Y, seven of A138V, one of A138T, and four of E192K. To confirm that no other extraneous mutations had occurred, the entire gene was sequenced for four representative isolates. All four mutants still bore the original TS6 mutation and of these, three contained a single additional change. In the remaining case, two coding changes were found, A138T and V166I. The A138T mutation was shown to be responsible for the SOS inducing phenotype and further descriptions of this allele (B216) refer to the A138T mutation alone in the TS6 background.

Table I describes the phenotypes observed when these *Eco*RI mutants are expressed in the SOS-*lacZ* fusion strain JH137 bearing the *Eco*RI methylase plasmid (pJC1). As expected, the parent TS6 mutant does not make blue colonies at any temperature because its activity is blocked by the *Eco*RI methylase. At 42°C where the TS endonuclease is largely inactive, the mutant enzymes induce little or no SOS response. As the temperature is decreased to activate endonuclease action, the SOS response is induced even though the *Eco*RI methylase is present. Although the mutants damage the DNA of the host cell and induce the SOS response, only the B216 mutant is fully lethal at 30°C where endonuclease activity should be maximal. The mutant endonucleases are lethal at 30°C when expressed in the absence of the *Eco*RI methylase (data not shown), indicating that the *Eco*RI methylase partially protects the cell from their action. In two cases, mutants were recombined together *in vitro*; the resulting triple mutants (L2=E192K + A138V + R56Q: L4=E192K + H114Y + R56Q) are more active than their parents and kill the cell at 30°C even when the *Eco*RI methylase is present (Table I). Thus the phenotypes of the mutations are additive. Lastly, for at least two mutants, the TS6 mutation does not play a role in the mutant phenotype, because the E192K and Y193H mutations (described below) still induced the SOS response when isolated in an otherwise wild-type background. In addition, the isolated E192K and Y193H mutations induced the SOS response at all temperatures and thus do themselves render the enzyme TS. Collectively, these *in vivo* observations

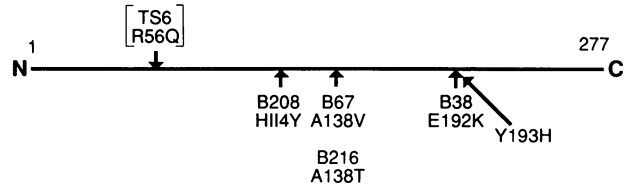

Fig. 1. Positions of amino acid substitutions that allow the *Eco*RI endonuclease to evade the *Eco*RI methylase. As described in the text, we screened for *Eco*RI endonuclease mutants that would damage the DNA of the host cell and induce the SOS response even though the *Eco*RI methylase was present. With the exception of the Y193H mutation, each mutant bears two amino acid substitutions: the R56Q temperature-sensitive mutation (shown in brackets) and another indicated amino acid change. The Y193H star activity mutation was isolated as a spontaneous suppressor of the *Eco*RI substrate binding pocket mutation R145K (see results).

Table I. *Eco*RI mutants which induce SOS even in the presence of the methylase

Temp.	Allele							
	WT	TS6	B38	B67	L2*	B208	L4**	B216
42°C	W-LB	W-LB	W-LB	LB	W-LB	W-LB	W-LB	LB
37°C	W-LB	W-LB	LB	MB	LB	LB-MB	LB	MB-DB
34°C	W-LB	W-LB	LB-MB	MB	DB	DB, sick	MB-DB	DB, sick
30°C	W-LB	W-LB	LB	MB	dead	DB, sick	dead	dead
Mutations		R56Q	R56Q	R56Q	R56Q	R56Q	R56Q	R56Q
			E192K		E192K		E192K	
				A138V	A138V			A138T
						H114Y	H114Y	

*L2=B38 + B67
**L4=B38 + B208
SOS induction was assayed by measuring β-galactosidase production (using X-Gal indicator medium) from the *dinD1*::Mu dI(Ap^r *lac*) fusion borne by strain JH137/pJC1.
W=white colonies on X-gal (35 μg/ml) indicator medium 0–10 units β-galactosidase
LB=light blue, 10–60 U β-galactosidase
MB=medium blue, 60–100 U β-galactosidase
DB=dark blue, 100+ U β-galactosidase
dead=no colonies
sick=small, mottled, poorly growing colonies

suggest that these *Eco*RI mutant enzymes retain some ability to cleave the wild-type substrate, but have also gained the ability to cleave sites not protected by the *Eco*RI methylase.

Cleavage specificity of the mutant enzymes

To determine the *in vitro* cleavage activity that corresponds to the *in vivo* phenotype, the mutant *Eco*RI enzymes were purified (see Materials and methods) and DNA digestions were performed with substrates of known sequence. While the wild-type and TS6 mutant enzymes cleave plasmid pBR322 at its *Eco*RI site (aGAATTCt) to yield one linear species (Figure 2, lanes b,c), the mutant proteins generate fragments of ~1600 and ~2400 bp which correspond to fragments produced by the *Eco*RI* activity of the wild-type enzyme (compare lanes d–i with lane j in Figure 2). By appropriate double digests (data not shown), we find that the mutant proteins preferentially cleave at the wild-type *Eco*RI recognition site, and more slowly (about 10-fold) at a second site which maps to the position of a previously described *Eco*RI* site, gGAAGTCa (nucleotides 1636–1643) (Gardner *et al.*, 1982). The mutant enzymes also cleave an *Eco*RI* site present on plasmid pACYC177 (tGACTTCa, nucleotides 955–962; Rose, 1988) (data not shown).

When plasmid pBR322 DNA is incubated with these mutant enzymes in *Eco*RI* buffer, cleavage occurs at a number of additional sites (Figure 2, lanes k–q). An identical pattern of fragments is observed after prolonged digestion with the wild-type enzyme in *Eco*RI* buffer (data not shown). We conclude that these mutants exhibit enhanced cleavage activity at *Eco*RI* sites under reaction conditions where the wild-type enzyme exhibits no *Eco*RI* action and that the activity of the mutant enzymes is increased by *Eco*RI* buffer. From the pattern of partial digestion (Figure 2, lanes m and p), it appears that the B67 and B216 mutant enzymes cleave some *Eco*RI* sites less readily than others, suggesting that the mutants may differ in substrate preference.

We turned to f1 DNA as a substrate because it has no wild-type *Eco*RI sites and contains more *Eco*RI* sites than pBR322. Figure 3A shows partial digestions of f1 RFI form DNA treated with the wild-type *Eco*RI enzyme in *Eco*RI* buffer and with the L2 mutant enzyme (R56Q + A138V + E192K) in standard *Eco*RI buffer. Although f1 contains no *Eco*RI sites, it is nonetheless nicked at several *Eco*RI* sites when incubated with the wild-type *Eco*RI enzyme in standard buffer conditions (Figure 3A, lane c; see also Heitman, 1989). As shown in lanes l–r of Figure 3A, the *Eco*RI* activity of the wild-type enzyme slowly cleaves f1. In marked contrast, the L2 mutant enzyme cleaves rapidly to yield three prominent fragments (Figure 3A, lanes f–k). With prolonged incubation, several additional sites are cleaved to yield fragments which in some cases co-migrate with *Eco*RI* products (Figure 3B, compare lanes 6 and 7). By double digests and scission of small purified restriction fragments, we mapped these cleavage sites to within

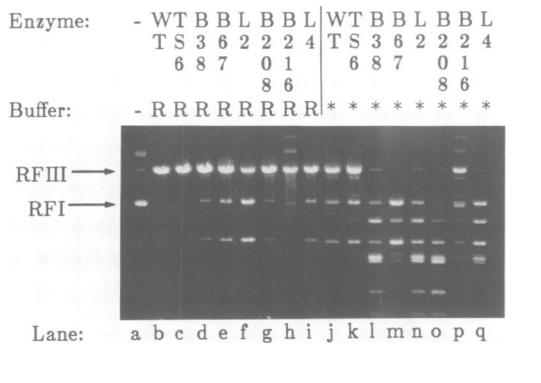

Fig. 2. Cleavage of pBR322 DNA by the wild-type and star mutant endonucleases. Plasmid pBR322 DNA (200 ng per reaction) was incubated with the purified wild-type or star mutant endonucleases for 1 h at 30°C in either standard *Eco*RI buffer conditions (lanes b–i) or *Eco*RI* buffer conditions (lanes j–q). The cleavage products were then electrophoresed through a 0.6% agarose gel containing 0.5 μg/ml EtBr. The difference in salt concentration between standard *Eco*RI and *Eco*RI* buffer gives rise to the slight mobility difference observed for the cleavage products in lanes b–i compared with those in lanes j–q. In the reaction electrophoresed in lane h, the DNA species migrating faster than linear plasmid DNA (RFIII) is uncleaved plasmid DNA (RFI).

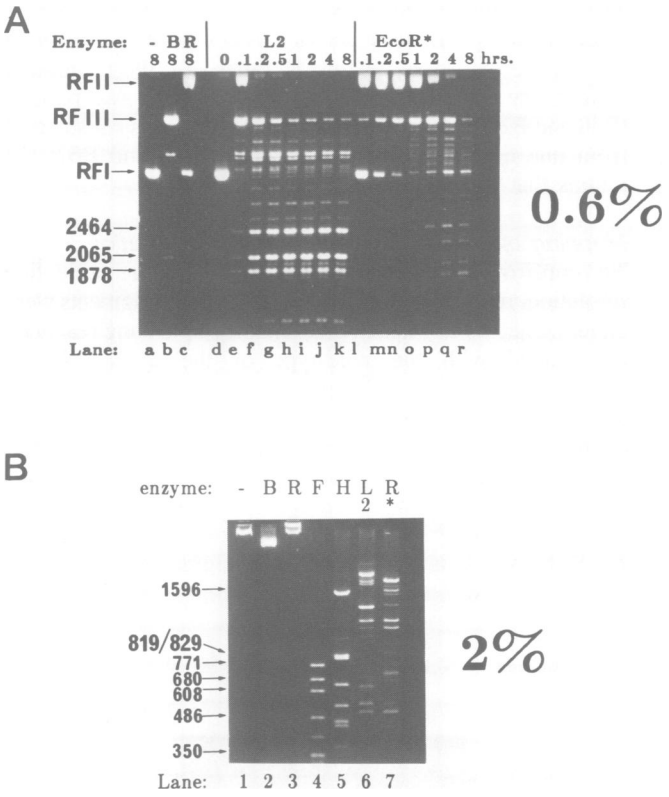

Fig. 3. Restriction digests of f1 DNA with the star mutant L2 or *Eco*RI* activity. **Panel A.** Lane a shows untreated supercoiled f1 DNA, lane b the linear species (cleaved at the one *Bam*HI site), and lane c the nicked product observed when f1 DNA is incubated with the wild-type *Eco*RI endonuclease in standard *Eco*RI buffer conditions. Each lane contains 250 ng f1 DNA. Lanes d–k and l–r show f1 partial digests with either *Eco*RI* or the L2 mutant enzyme. For lanes d–k, 2 μg of f1 DNA were incubated with 25 ng of the purified L2 mutant protein in 400 μl of standard *Eco*RI buffer at 30°C. At the indicated times, 250 ng of the DNA cleavage products were removed and the reaction was terminated with stop buffer. For the reactions shown in lanes l–r, 2 μg f1 DNA were incubated with 62.5 ng of the wild-type enzyme in *Eco*RI* buffer. Cleavage products were displayed on a 0.6% agarose gel containing 0.5 μg/ml EtBr. **Panel B.** To produce a complete restriction digest, 250 ng of f1 DNA were incubated with the indicated enzymes for 8 h at 30°C and the reactions were electrophoresed through a 2% agarose gel. Several of the bands resulting from cleavage by the L2 mutant protein (lane 6) co-migrate with fragments produced by the *Eco*RI* activity of the wild-type enzyme (lane 7) B=*Bam*HI; R=*Eco*RI; F=*Hin*f1; H=*Hpa*II.

10–20 bp (data not shown). By comparison to the known sequence of f1 (Hill and Petersen, 1982), we find that each interval contains a consensus *Eco*RI* site. These *Eco*RI* sites were demonstrated to be the sites of cleavage as described below (summarized in Figure 6).

As shown in Figure 4, a pattern of preferential cleavage similar to that of the L2 mutant was also observed with the B67 (R56Q + A138V) and B216 (R56Q + A138T) star mutant enzymes. Compared to these star mutant proteins (L2, B216, B67), DNA scission by the wild-type *Eco*RI enzyme in star buffer is slower, does not produce the three prominent fragments observed with the mutant proteins, and yields a more complex pattern of partial digestion. This suggests that the wild-type enzyme in *Eco*RI* conditions cleaves at more sites than the mutant enzymes and with lower specific activity. In contrast to the B67, B216, and L2 mutants, the B38 (R56Q + E192K), B208 (R56Q + H114Y), and L4 (R56Q + H114Y + E192K) star mutant enzymes yielded a partial pattern of digestion very similar to the wild-type *Eco*RI* activity (see Figure 4). We note that for the two triple mutants (L2 and L4), the L4 mutant exhibits the substrate specificity shared by the two parents (B38 and B208), whereas the L2 triple mutant is constructed from one mutant of either specificity (B38 and B67) yet retains that of one parent (B67).

Mapping cleavage sites at nucleotide resolution

To confirm these cleavage sites at single nucleotide resolution, uniquely end-labeled f1 restriction fragments were either subjected to Maxam and Gilbert sequencing reactions or cleaved with the wild-type or the mutant *Eco*RI endonucleases. The reaction products were displayed on both sequencing and non-denaturing polyacrylamide gels. One example is shown in Figure 5. A 193 bp *Cla*I–*Aha*III fragment spanning a site at position 6125 (GAATTT) yields an 88 bp fragment when cleaved by either the wild-type enzyme in *Eco*RI* buffer or the mutant enzymes in standard *Eco*RI buffer. This cleavage product migrates with the same

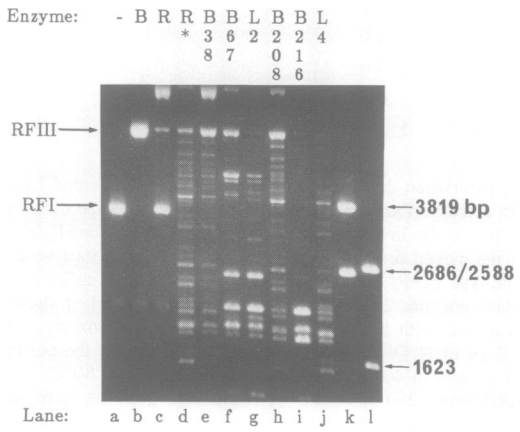

Fig. 4. Restriction digests of f1 DNA with *Eco*RI star mutant enzymes. 400 ng of CsCl purified RFI form DNA were incubated with the purified wild-type or star mutant *Eco*RI enzymes (25–75 ng) for 1 h at 30°C in standard *Eco*RI buffer (except lane d=wild-type *Eco*RI in star (*) buffer). Reaction products were electrophoresed through a 0.6% agarose gel containing 0.5 μg/ml EtBr. B=*Bam*HI, R=*Eco*RI wild-type. Size markers were an f1 *Bam*HI+*Cla*I double digest (lane k) and an f1 *Hae*III digest (lane l).

or slightly slower mobility than the fragment corresponding to formic acid cleavage at the first adenine within the sequence GAATTT, demonstrating that scission occurs between the guanine and the adenine as expected. Scission by either the wild-type or the mutant *Eco*RI enzymes produces a 3′-hydroxyl terminus because the enzymatic cleavage product migrates ~0.5 nucleotides slower than the Maxam and Gilbert cleavage product which bears a 3′-phosphoryl terminus. The 88 bp fragment was still observed when the reaction products were electrophoresed in non-denaturing gels, indicating that both DNA strands are cleaved (Figure 5B). Additional cleavage sites were mapped in the same way and are summarized on the f1 map in Figure 6.

Substrate specificity of the EcoRI mutant enzymes

A consideration of the mapped cleavage sites reveals that the mutant enzymes accept as substrates the wild-type site and *Eco*RI* sites containing either an adenine instead of guanine at the first position (AAATTC=GAATTT) or a substitution of the internal adenine by a cytosine (GACTTC=GAAGTC). The wild-type *Eco*RI* activity tolerates other single base substitutions (Rosenberg and Greene, 1982); however, in general these two sequences are the most readily cleaved *Eco*RI* sites (Rosenberg and Greene, 1982; Thielking *et al.*, 1990). For the three mutant enzymes that yield a partial cleavage pattern different from the wild-type enzyme in *Eco*RI* buffer (B216, B67, L2), the three preferential cleavage sites are all flanked by a thymidine at the 5′-end and an adenine at the 3′-end, the moderately cleaved sites have one or the other of these flanking nucleotides but not both, and the two poorest cleavage sites have neither. Although scission by the wild-type *Eco*RI enzyme shows some preference for sites flanked by AT base pairs (Thomas and Davis, 1975; McLaughlin *et al.*, 1987), we do not observe as marked an effect with wild-type *Eco*RI* activity compared to these star mutant enzymes (see Figure 4).

Enhanced star activity mutations suppress EcoRI binding site mutations

In a study of the *Eco*RI substrate binding pocket, a large number of site-directed mutations at amino acids E144, R145, and R200 were generated (Heitman, 1989; Heitman *et al.*, 1989c; Heitman and Model, 1990). The purified mutant enzymes are reduced in specific activity but retain specificity for the wild-type substrate. Two observations suggested that conditions which promote *Eco*RI* activity enhance the catalytic activity of these site-directed mutants. First, *Eco*RI* buffer conditions increase DNA scission at the wild-type site by the purified R200K and R200C mutant enzymes. Second, during these site-directed mutagenesis studies we found two isolates of the R145K substitution with different levels of enzyme activity, based on their ability to sponsor SOS induction in strain JH137 (lacking the *Eco*RI methylase). DNA sequencing of the entire gene of both mutants revealed an additional mutation in the more active allele. This spontaneous second-site suppressor results from an amino acid substitution (Y193H) that lies adjacent to a residue affected by one of the star activity mutations (E192K). To determine the phenotype of the Y193H mutation alone, the R145K mutation was changed to the wild-type amino acid (arginine) by site-directed reversion. The mutant bearing only the Y193H mutation promotes a weak

but significant SOS induction in cells expressing the *Eco*RI methylase (strain JH137/pJC1). Although this mutant protein has not been characterized *in vitro*, based on the location of the mutation, and phenotype similar to the star mutants, it seems likely that the Y193H mutation represents a fifth star mutation.

The finding that the Y193H mutation increased the weak endonuclease activity of the R145K mutant protein suggested that the star mutations might in general suppress other substrate binding pocket mutations. By taking advantage of a convenient *Bgl*II site, most of the possible amino acid substitutions of R200 were recombined together with the A138V, H114Y, or A138T star mutation. The R200X mutations in the TS6 mutant background (double mutants) were compared to triple mutants which also carry the *Eco*RI star mutations. Endonuclease activity was assessed by monitoring β-galactosidase expression (on X-gal indicator medium) from the SOS::*lacZ* fusion in strains JH137 (lacking the *Eco*RI methylase) and JH137/pJC1 (expressing the *Eco*RI methylase). As shown in Table II, star mutations suppress the decreased activity phenotype conferred by the R200C, V and S mutations leading to a greater induction of the SOS response (in strain JH137). In contrast, the SOS response was not induced when the *Eco*RI methylase was present, demonstrating that suppression increases endonuclease activity at only the wild-type *Eco*RI site. The R200K mutant was not suppressed by any of the star mutations.

By the criteria of suppression by star mutations, null R200X mutants fell into two classes (see Table II). Star mutations did not suppress the cleavage defect of mutants bearing leucine, glycine, asparagine or glutamine at position 200. In contrast, when mutants containing methionine, threonine, alanine or isoleucine were recombined with star mutations, endonuclease activity was readily detectable by its ability to induce the SOS response in strain JH137. In general, the star mutations suppress R200X null mutants which bear amino acid substitutions structurally similar to the active R200X mutants (serine, cysteine, or valine). In each of these cases, endonuclease activity (SOS induction) was again blocked by the *Eco*RI methylase and is therefore specific for the canonical *Eco*RI substrate. We conclude that the enhanced star activity mutations act as suppressors of substrate pocket mutations. In summary, the star mutations increase catalytic activity of the *Eco*RI enzyme in two cases: they allow an otherwise wild-type enzyme to cleave substrates (*Eco*RI* sites) lacking functional groups normally required for substrate recognition and catalysis, and they permit the *Eco*RI substrate binding pocket mutants to cleave the wild-type substrate better in the absence of functional groups normally provided by the enzyme.

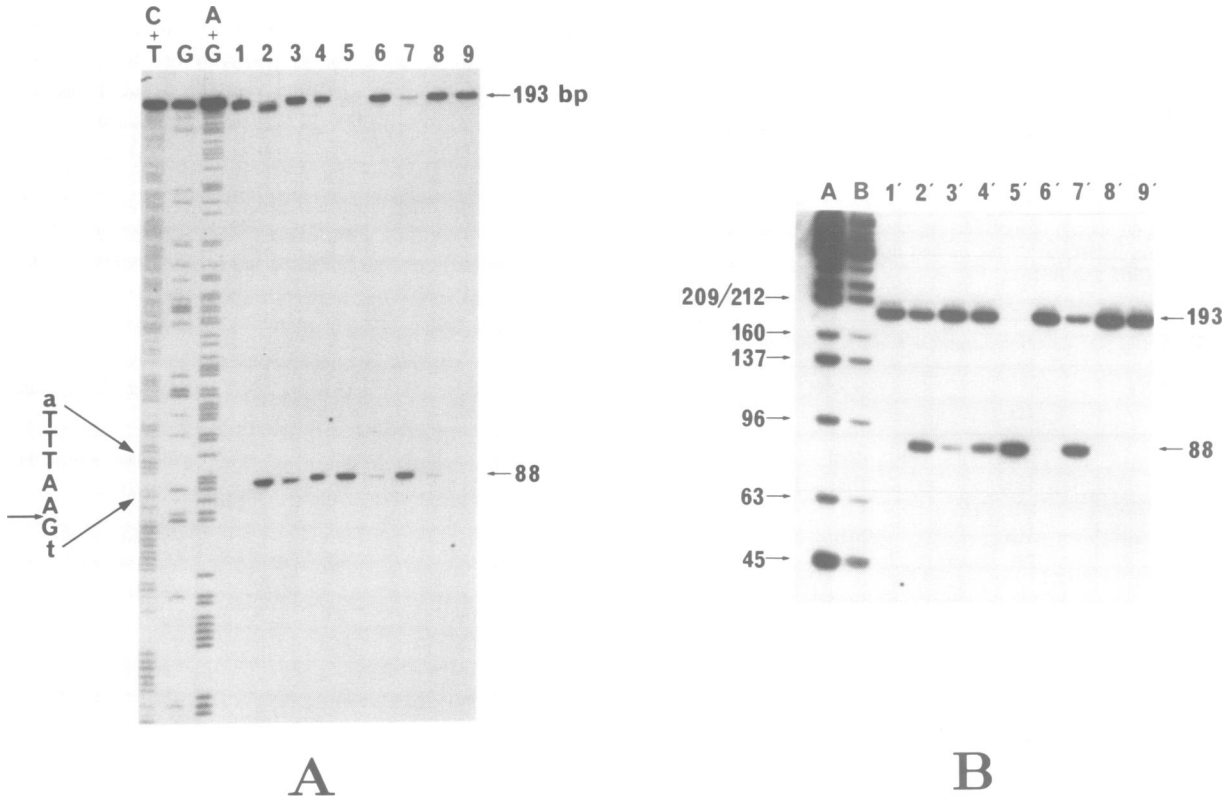

Fig. 5. Mapping star mutant cleavage sites to single nucleotide resolution. **Panel A.** A 193 bp *Cla*I–*Aha*III fragment which spans the recognition site at position 6125 was cleaved with the wild-type or mutant *Eco*RI enzymes and the reaction products were electrophoresed through a 6% polyacrylamide–8M urea sequencing gel. The left three lanes are the Maxam and Gilbert chemical sequencing reactions. Lane 9 shows the uncleaved sample, while lanes 1–8 are restriction digests with the wild-type or mutant enzymes. Scission occurs at one site to yield an 88 bp product, which is attributable to cleavage at the indicated position (arrow) in the sequence written to the left. Lanes: 1=wild-type enzyme in standard *Eco*RI buffer; 2=wild-type enzyme in star buffer; lanes 3–8 were all in standard *Eco*RI buffer; lane 3=B38; lane 4=B67; lane 5=L2; lane 6=B208; lane 7=B216; lane 8=L4. **Panel B.** The same restriction digests were electrophoresed through an 8% non-denaturing polyacrylamide gel. Lanes A and B are ^{32}P-end-labeled *Hin*fI restriction fragments of f1 as size markers. Lane 1′ to 9′ correspond to the same reactions shown in lanes 1–9 of panel A. The same 88 bp cleavage product is still observed, indicating that both DNA strands have been cleaved.

Discussion

We have isolated and characterized *Eco*RI endonuclease mutants that cleave the DNA of their host cell despite the presence of the *Eco*RI methylase. *In vitro*, the purified mutant enzymes preferentially cleave the wild-type recognition site with specific activity within an order of magnitude of the wild-type enzyme (data not shown). When incubated in standard *Eco*RI buffer conditions, these mutant proteins also cleave *Eco*RI* sites that differ by one nucleotide from the canonical substrate. The mutant enzymes yield a restriction pattern which is either identical to that produced by the *Eco*RI* activity of the wild-type enzyme or the result of preferential scission at a subset of *Eco*RI* sites with particular flanking sequences. In contrast, in standard *Eco*RI buffer, the wild-type enzyme cleaves the wild-type substrate with a 10^4-fold (Thielking *et al.*, 1990) to 10^7-fold (Halford and Johnson, 1980) preference over other sites. The wild-type enzyme fully cleaves *Eco*RI* sites to a significant extent only when incubated in *Eco*RI* buffer conditions (Polisky *et al.*, 1975; Hsu and Berg, 1978; Malyguine *et al.*, 1980; Gardner *et al.*, 1982; Rosenberg and Greene, 1982). We (see Figure 4) and others (Thielking *et al.*, 1990) have observed that some *Eco*RI* sites are nicked by the wild-type enzyme under standard buffer conditions. We note that the intracellular ionic conditions within the *E.coli* cell (Leirmo *et al.*, 1987) are such that the wild-type protein should not fully cleave *Eco*RI* sites *in vivo*, but some *Eco*RI* sites could be nicked *in vivo* as observed *in vitro*. We observe little or no SOS induction by the wild-type *Eco*RI restriction–modification system, and thus *in vivo* nicks at *Eco*RI* sites may be rare, rapidly repaired by ligation, or poor inducers of the SOS response. In summary, we conclude that the star mutants escape the protective action of the *Eco*RI methylase *in vivo* because they have increased *Eco*RI* activity. That mutations can increase *Eco*RI* activity suggests that *Eco*RI* buffer exerts at least part of its effects by perturbing the structure or the action of the *Eco*RI enzyme.

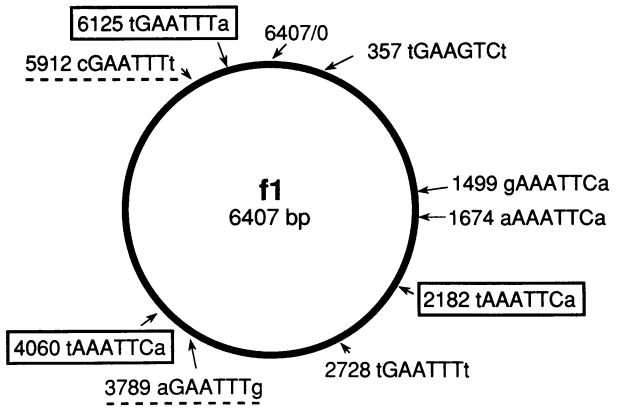

Fig. 6. Sites of DNA scission on the f1 map. The sites at which the star mutant endonucleases cleave f1 are indicated. These sites are all *Eco*RI* sites and are cleaved by the wild-type enzyme under *Eco*RI* conditions. The B38, B208, and L4 star mutants cleave to yield a restriction pattern identical to that observed with the *Eco*RI* activity of the wild-type enzyme. In contrast, the B67, L2, and B216 mutant enzymes preferentially cleave a subset of *Eco*RI* sites more readily than the *Eco*RI* activity of the wild-type enzyme. For these mutant proteins, the boxed sites represent the three most rapidly cleaved sites, the sites with no special designation are cleaved at an intermediate rate, and those underlined by the dashed line are cleaved slowly (L2) or not at all (B67 and B216).

The star mutations also suppress the cleavage defect of *Eco*RI mutants (such as R200C, R200V, and R200S) altered at amino acids in the DNA binding domain. In contrast to other R200X alleles, the R200K mutant was not suppressed by the star mutations (Table II). This is surprising because the R200K mutant protein is activated 100-fold by *Eco*RI* buffer (Heitman, 1989; Heitman and Model, 1990). These observations suggest that the cleavage defect of the R200K allele is functionally different from that of the R200C, R200V or R200S mutants. Because the R200 residue is thought to form salt bridges that stabilize the dimer interface (McClarin

Table II. *Eco*RI star mutations suppress substrate binding site mutations

Allele (R200X) X=		+R56Q	+R56Q +A138V	+R56Q +H114Y	+R56Q +A138T	Effect of star mutations
R (WT)	dead	dead	dead	dead	dead	increase star activity
K	dead	DB, sick	DB, sick	DB, sick	DB, sick	no effect
C	DB, sick	LB	DB, sick	dead	dead	increase wild-type activity
V	MB	MB	dead	dead	dead	increase wild-type activity
S	LB	W-LB	MB	MB	DB, sick	increase wild-type activity
T	W	W	LB	LB-MB	MB	render mutant active
M	W	W	MB	W-LB	MB	render mutant active
A	W	W	LB	W-LB	LB	render mutant active
I	W	W	LB	W-LB	LB	render mutant active
L	W	W	W	W	W	no effect
G	W	W	W	W	W	no effect
N	W	W	W	W	W	no effect
Q	W	W	W	W	W	no effect

Indicator medium contained 35 μg/ml X-gal.
The host was strain JH137. Growth was at 30°C.
W=white colonies, 0–10 U β-galactosidase
LB=light blue colonies, 10–60 U β-galactosidase
MB=medium blue colonies, 60–100 U β-galactosidase
DB=dark blue colonies, 100+ U β-galactosidase.
dead=no colonies
sick=small, mottled, poorly growing colonies

et al., 1986; Geiger *et al.*, 1989), the conservative lysine substitution in the R200K mutant may maintain the local electrostatic configuration of the protein while the cysteine, valine, and serine substitutions do not.

The recently determined sequence of an *Eco*RI isoschizomer, *Rsr*I, revealed that these two enzymes share 50% homology (Stephenson *et al.*, 1989). One therefore suspects that residues critical for enzyme function have been conserved, and correspondingly that conserved residues may be more critical than those not conserved. In fact, of the residues implicated in *Eco*RI substrate recognition (McClarin *et al.*, 1986) and catalysis (King *et al.*, 1989; Wright *et al.*, 1989), all are shared by the two enzymes ($E111_{EcoRI}$=$E116_{RsrI}$, $E144_{EcoRI}$=$E149_{RsrI}$, $R145_{EcoRI}$=$R150_{RsrI}$, $R200_{EcoRI}$=$R205_{RsrI}$, $R203_{EcoRI}$=$R208_{RsrI}$, the homology between *Eco*RI and *Rsr*I is observed when the sequences are displaced by five residues because *Rsr*I has five additional N-terminal amino acids not found in *Eco*RI). Of the *Eco*RI star mutants described here, two of the residues present in wild-type *Eco*RI are conserved in *Rsr*I ($H114_{EcoRI}$=$H119_{RsrI}$, and $A138_{EcoRI}$=$A143_{RsrI}$) whereas two others are not ($E192_{EcoRI}$=$V197_{RsrI}$ and $Y193_{EcoRI}$=$H198_{RsrI}$). This comparison suggests that residues H114 and A138 are vital for *Eco*RI function while residues E192 and Y193 are less so. This is in keeping with our observation that the H114Y, A138V and A138T star mutations dramatically affect *Eco*RI activity while the phenotypes of the E192K and Y193H mutants are more subtle (Table I and Results). Interestingly, one of the amino acids substitutions (Y193H) that increases the star activity of *Eco*RI is found in the wild-type *Rsr*I enzyme ($H198_{RsrI}$), suggesting that *Rsr*I could have increased star activity compared to *Eco*RI unless other amino acid differences are compensatory. In addition, an amino acid substitution that renders the *Eco*RI enzyme TS (R56Q=TS6; Heitman and Model, 1989a) is also found in the wild-type *Rsr*I enzyme ($Q61_{RsrI}$), and this may account for the temperature-sensitive activity of *Rsr*I (Aiken and Gumport, 1988; Stephenson *et al.*, 1989).

By our genetic approach we have identified four residues of *Eco*RI (H114, A138, E192, Y193) that are involved in DNA recognition and catalysis. Notably these amino acids are distinct from those which the *Eco*RI–DNA co-crystal structure (McClarin *et al.*, 1986) implicated as playing a role in substrate recognition (E144, R145, R200). Because the *Eco*RI crystal structure has recently been found to be incorrect in some features (J.Rosenberg, personal communication), a full analysis of the structural implications of these *Eco*RI star mutants must await a revised structure. These mutations must however affect substrate recognition by altering or perturbing one of four features of the DNA–protein complex: direct DNA base–protein contacts, phosphate–protein interactions, the enzyme active site, or the allosteric machinery proposed to couple DNA binding and substrate recognition. We now consider each of these possibilities.

The model derived from the original *Eco*RI crystal structure stressed that substrate recognition is mediated by hydrogen bonds with only the purines of the substrate. However, based on our genetic findings (Heitman *et al.*, 1989c; Heitman and Model, 1990), we proposed an alternative in which the enzyme also contacts the substrate pyrimidines and thus recognizes both members of each basepair (Heitman and Model, 1990). In addition to our genetic data, the pyrimidine contact model is based on biochemical studies that revealed scission by *Eco*RI is inhibited or prevented by C^5-methylation of the substrate cytosines or substitution of thymidine by uracil (especially at the outer thymidines) to delete the thymidine 5-methyl group (Brennan *et al.*, 1986; McLaughlin *et al.*, 1987; Tasseron-de Jong *et al.*, 1988). The substitution of thymidine by uracil has no (GAATUC) or only minor (GAAUTC) effects on the curvature of the *Eco*RI site (Diekmann and McLaughlin, 1988), arguing that these modifications do not affect *Eco*RI action indirectly. The pyrimidine contact model is also consistent with both the observation that *Eco*RI binding inhibits photofootprinting of the substrate pyrimidines (Becker *et al.*, 1988) and a recent study of *Eco*RI sites containing basepair mismatches (Thielking *et al.*, 1990). For example, a site containing an AC mismatch at position 1 of the *Eco*RI site is a much better substrate than an *Eco*RI* site in which an AT basepair replaces the wild-type GC basepair at position 1, suggesting that the wild-type pyrimidine base present in the mismatch contributes to substrate recognition and improves catalysis of the mismatched substrate. This would be consistent with a direct pyrimidine–protein contact, but does not exclude the possibility that backbone distortion at the mismatch promotes catalysis by resembling the kinked DNA observed in the *Eco*RI–DNA crystal structure (Frederick *et al.*, 1984). Collectively these observations provide compelling support for the pyrimidine contact model in which the *Eco*RI enzyme makes hydrophobic or steric contacts to pyrimidines that contribute to substrate recognition. We suggest that pyrimidine interactions may appear in the revised version of the *Eco*RI–DNA crystal structure.

In terms of the pyrimidine contact model, we consider that the star mutations altering residue A138 (A138V, A138T) are those most likely to affect a direct DNA–protein interaction. In the original *Eco*RI crystal structure (McClarin *et al.*, 1986), residue 138 lay in a loop of protein within the DNA major groove. Moreover, alanine is a hydrophobic amino acid and in at least one other case (a mutant 434 repressor), it contacts the 5-methyl group of a thymidine (Wharton and Ptashne, 1987). We suggest that A138 either makes a direct DNA–protein contact, or participates with adjacent hydrophobic residues (M137, A139) to form a hydrophobic DNA recognition surface that discerns the substrate pyrimidine(s). According to this model the A138V and A138T mutations either directly alter a DNA–protein interaction or perturb neighboring residues that bind DNA. Interestingly, the analogous mutations have been isolated in the adjacent residue, A139 (Yanofsky *et al.*, 1987). Unlike the A138V and A138T mutant proteins, the A139V and A139T mutant enzymes are not lethal when conditionally expressed in the absence of the *Eco*RI methylase, suggesting that A139V and A139T are null mutants although it would now be of interest to reassess their activity at lower temperatures and with a more sensitive assay (SOS induction). These additional mutations further implicate the region around residue 138 as critical for *Eco*RI activity.

In addition to direct DNA recognition, these *Eco*RI star mutations may affect interactions with the DNA sugar–phosphate backbone (indirect DNA recognition). For example, two star mutations (A138V, A138T) result in mutant enzymes whose activity at *Eco*RI star sites is very sensitive to flanking sequences. Residue 138 lies within a

portion of the protein that lies near the substrate, thus this region of the protein may modulate or participate in recognition of the edges of the substrate. Flanking nucleotides may affect the ability of the DNA to form the kinks observed in the crystal structure at both the edges and the center of the *Eco*RI site (Frederick *et al.*, 1984). This is probably an example of indirect sequence recognition, in which specific DNA sequences alter the structure of the sugar phosphate backbone and thereby affect DNA–protein interactions (Dickerson, 1983; Otwinowski *et al.*, 1988). Of the five isolated *Eco*RI star mutants, two result in the addition of positive charges (E192K, Y193H) while another removes a positive charge (H114Y). Should any of these residues lie at the DNA–protein interface (as do both E192K and Y193H in the original *Eco*RI crystal structure: McClarin *et al.*, 1986), these mutations would alter positive charges near the negatively charged phosphate backbone of the DNA and could change the electrostatic DNA binding surface and impair substrate recognition. By ethylation interference studies, the wild-type *Eco*RI enzyme makes very different phosphate contacts when bound to wild-type versus *Eco*RI star sites (Lesser *et al.*, 1990). Thus, mutations that increase *Eco*RI star activity might do so by permitting the enzyme to bind *Eco*RI star sites and make phosphate contacts normally observed only at wild-type *Eco*RI sites.

Little is known about the structure and function of the *Eco*RI active site. As has been found for other enzymes, *Eco*RI substrate recognition and catalysis may not be entirely separable. For example, certain mutations in the subtilisin catalytic triad residues alter substrate specificity while others at substrate binding amino acids decrease catalysis (Estell *et al.*, 1986; Carter and Wells, 1988). Similarly, we found that mutating putative substrate binding residues of *Eco*RI dramatically reduced enzyme activity without altering substrate specificity (Heitman and Model, 1990). Thus substrate recognition and catalytic residues are inextricably linked. One might therefore expect to find that some mutations that change substrate specificity do so indirectly, by affecting the structure of the active site. By this reasoning, the *Eco*RI star mutations could increase catalysis by the active site or uncouple the active site and recognition domains such that substrate fidelity is decreased and *Eco*RI* sites are cleaved.

Lastly, these *Eco*RI* mutations could affect an allosteric activation mechanism that couples *Eco*RI binding and cleavage (McClarin *et al.*, 1986; Terry *et al.*, 1987). The *Eco*RI allosteric activation model is based on the co-crystal structure (McClarin *et al.*, 1986), kinetic and thermodynamic analyses (Terry *et al.*, 1987; Ha *et al.*, 1989), and the phenotypes of mutant enzymes (Jen-Jacobson *et al.*, 1983). The crystal structure of an *Eco*RI–DNA complex revealed that the enzyme bears arms that enwrap the DNA tightly. After DNA binding, these arms must be flexible enough to change conformation and embrace the DNA. By kinetic analysis, Terry *et al.* (1987) found that during a catalytic cycle the *Eco*RI enzyme spends most of its time bound to non-specific DNA sequences which are rarely cleaved. They proposed that the catalytic center is inactive in the non-specific complex and only becomes active upon substrate binding. Ha *et al.* (1989) examined the thermodynamics of the *Eco*RI–DNA interaction and found that a larger hydrophobic surface is buried than would be predicted by the crystal structure of chemical probe studies. They suggested that additional non-polar surfaces of *Eco*RI are removed from solvent by conformational changes promoted by sequence-specific binding. Lastly, a spontaneous *Eco*RI mutant (R187S) with decreased activity and impaired DNA binding has been described (Jen-Jacobson *et al.*, 1983). *In vitro*, the mutant enzyme makes only two ionic contacts to the DNA at pH 7.4 and six at pH 6, in contrast to eight ionic contacts for the wild-type enzyme at either pH. The R187S mutation may disrupt conformational changes that bring other amino acids into ionic contact with the DNA (Jen-Jacobson *et al.*, 1983).

In terms of the allosteric activation model, the mutations described here could increase *Eco*RI* activity by promoting the allosteric conformational changes that are initiated by substrate binding and subsequently transmitted to the active site to trigger DNA cleavage. This view is supported by our observation that the decreased activity of *Eco*RI mutants bearing substitutions in the substrate binding pocket (R145K and R200X) is suppressed by mutations that increase star activity of the wild-type protein. According to this model, mutations in the substrate binding domain hinder allosteric activation whereas the star mutations promote activation. Star mutations that alter residues within the substrate binding domain may permit *Eco*RI star sites to trigger transmission of the activating signal, perhaps, for example, by altering the conformational change involving the arm. On the other hand, mutations at residues near or in the active site could alter reception of the activating signal. Interestingly, *Eco*RI* buffer increases the specific activity of the star mutant enzymes, suggesting that *Eco*RI* buffer may also promote allosteric activation of the *Eco*RI enzyme (this notion has also been suggested by King *et al.*, 1989). Lastly, the *Eco*RI star mutants may be similar to mutants of other proteins which are known to relax an allosteric effector requirement, such as the CAP* mutants (Garges and Adhya, 1985).

Many restriction enzymes, as well as the *Eco*RI methylase, exhibit reduced substrate specificity (star activity) when incubated with DNA in certain buffer conditions (Gardner *et al.*, 1982; George and Chirikjian, 1982; Nasri and Thomas, 1986; Barany, 1988; Berkner and Folk, 1978; Woodbury *et al.*, 1980a, 1980b). If mutations which enhance *Eco*RI* activity do so by affecting the *Eco*RI allosteric activation mechanism, restriction endonucleases and methylases may in general be allosterically activated upon substrate binding. By this model, restriction enzymes would achieve high fidelity substrate recognition through a two step mechanism, analogous to the sieve model for tRNA aminoacylation (Fersht, 1985). In the first step, the enzyme makes an extensive series of interactions that bind and discern the substrate. In the second, only the preferred substrate induces allosteric conformational changes that trigger scission and act as a final check on the fidelity of DNA cleavage.

Allosteric activation by DNA binding may be widespread and is not necessarily restricted to catalysis. In the arabinose operon of *E.coli*, some point mutations within the *araI* operator block transcriptional repression without altering the affinity with which the AraC protein binds *in vitro* (Martin *et al.*, 1986). This led to the proposal that transcriptional regulation by the AraC protein is allosterically coupled to sequence-specific DNA interactions (Huo *et al.*, 1988). Ha *et al.* (1989) concluded from thermodynamic considerations that many sequence-specific DNA binding proteins (*Eco*RI, *lac* repressor, *E.coli* RNA polymerase, P22 Mnt repressor)

change conformation upon binding DNA. In addition, specific DNA interactions alter the conformation of the yeast transcription regulator PRTF (or MCM1) (Tan and Richmond, 1990). Allosteric conformational changes induced by sequence specific interactions may be a general mechanism that modulates the activity of DNA binding proteins and monitors the fidelity of DNA sequence recognition.

Materials and methods

Bacterial strains

The bacterial strains used in this study are strains JH137 (=K38 Δ*lacZ dinD*::Mu dI (Ap^R *lacZ*) (Heitman and Model, 1987) and JM103 mutD (= *mutD* Δ*lacpro thi strA supE endA sbcB15/hsdR4/F'traD36 proAB lacI*^{q}ZΔ*M15*, supplied by J.Makris). SOS induction was assayed using a Mu dI (Ap^R *lacZ*) fusion to the *dinD* DNA damage inducible locus (Kenyon and Walker, 1980). β-galactosidase activity was monitored by growing colonies on YT plates supplemented with 35 μg/ml X-gal. β-galactosidase was assayed as described by Miller (1972).

Plasmid constructions

Plasmid pAN4 is a pBR322 derivative which encodes the *Eco*RI restriction−modification system and confers ampicillin resistance (Newman *et al.*, 1981). As described in Heitman *et al.* (1989a), the *Eco*RI methylase gene of pAN4 was inactivated to yield the R^+ M^- plasmid pJH10. Plasmid pJC1 is a pACYC184 derivative which encodes the *Eco*RI methylase and chloramphenicol resistance (Cheng and Modrich, 1983). Plasmids pJH15a and b carry the f1 intergenic region inserted as a cassette (Heitman *et al.*, 1989b) at the *Cla*I site of plasmid pJH10 such that replication from the f1+ strand origin is clockwise for pJH15a and counter-clockwise for pJH15b.

Deletions of the *Eco*RI endonuclease gene were constructed by inserting a *Bam*HI linker at the *Pvu*I site and an *Xho*I linker at the *Sma*I site of plasmid pJH15b. The resulting plasmid (pJH19) was partially cleaved with *Hin*dIII, *Bgl*II, or *Pst*I (sites internal to the *Eco*RI gene), treated with Klenow and dNTPs, ligated to *Bam*HI linkers, digested with *Bam*HI and religated. A number of R^-, Amp^R and Kan^R transformants were screened to yield the deletion plasmid pJH70 (deletes the N-terminal quarter of the endonuclease gene up to the *Hin*dIII site), pJH71 (removes the N-terminal half up to the *Bgl*II site), and pJH72 (removes the N-terminal three-quarters up to the *Pst*I site). An additional pJH15b deletion (pJH74, R^-, Amp^R, Kan^S) between the *Bgl*II sites in the endonuclease and kanamycin resistance genes removes the C-terminal half of the endonuclease gene.

Random mutagenesis and mutant screens

*Eco*RI endonuclease mutants with enhanced *Eco*RI* activity were isolated as follows. Independent cultures of strain JH137 bearing plasmids pJC1 (R^-M^+) and pJH15b (R^+M^-) carrying the TS6 allele were mutagenized *in vivo* with 25 or 50 μg/ml of nitrosoguanidine (Miller, 1972), and plasmid DNA was prepared after overnight growth. Alternatively, plasmid pJH15b (TS6) was mutagenized by growth in the *mutD* mutator host JM103 mutD. Strain JM103 mutD (pJC1 and pJH15b (TS6)) was first grown under conditions where the mutator activity is suppressed (minimal medium). Individual colonies were then grown overnight in LB medium to increase mutator activity (Cox and Horner, 1982) and plasmid DNA was prepared. Mutagenized plasmid DNA was introduced into the SOS::*lacZ* fusion strain JH137 expressing the *Eco*RI methylase, and the resulting transformants were plated on X-gal indicator medium and screened for blue colonies which were stable and TS for the blue colony phenotype.

Mapping and sequence analysis

Mutations were mapped by either of two methods. First, restriction fragments from wild-type and mutant plasmids were ligated and SOS induction by the hybrid was scored following transformation. Secondly, we employed heteroduplex deletion mapping (Shortle, 1983) with a set of *Eco*RI deletions. For this method, *Bam*HI linearized DNA of the *Eco*RI endonuclease deletion plasmids pJH70, pJH71 or pJH72, or *Bgl*II treated pJH74 was annealed to *Cla*I treated mutant DNA, the heteroduplex DNA introduced into strain JH137/pJC1, and transformants were scored for SOS induction (blue colonies) by plating on X-gal indicator medium at 30°C.

The implicated region for each mutation was sequenced by the dideoxy nucleotide method (Sanger *et al.*, 1977). In several cases (TS6, R145K, R145K + Y193H, and all the *Eco*RI star mutants), the entire gene was sequenced. Sequencing templates were either single-stranded plasmid DNA isolated after helper phage infection or denatured (collapsed) double-stranded plasmid DNA (Chen and Seeburg, 1985).

Site-directed mutagenesis

Site-directed mutagenesis to produce the E144X, R145X, and R200X mutants has been described (Heitman and Model, 1990). The R145K mutation, carried by the R145K + Y193H double mutant, was reverted by the method of Kunkel (1985) to yield the isolated Y193H mutation.

Protein purification

Two liter cultures (strain JH137 bearing both plasmid pJC1 and the wild-type or a mutant pJH15b plasmid) were grown at 42°C in FB media up to an OD_{600} of 0.2−0.6 and then shifted to 30°C for 10 h. The wild-type and mutant *Eco*RI enzymes were purified by phosphocellulose and hydroxyapatite chromatography as described by Cheng *et al.* (1984). By this means 100−200 μg of the wild-type enzyme, the TS6 parent, and the B38, B67, B208, B216, and L2 mutant enzymes were obtained. The yield for the L4 triple mutant was low (~20 μg), probably because of the extremely lethal effect of this mutant.

Enzyme assays and cleavage site mapping

Two buffers were employed for restriction digestions with the wild-type or mutant *Eco*RI endonucleases. Standard *Eco*RI buffer contains: 100 mM Tris−HCl, pH 7.5, 5mM $MgCl_2$, 50 mM NaCl and 100 μg/ml BSA. Eco-RI* (star) buffer contains: 25 mM Tris−HCl, pH 8.5, 2mM $MgCl_2$ and 5% glycerol (from the enzyme storage buffer). To map the f1 sites cleaved by the *Eco*RI star mutants, restriction fragments of f1 (*Hha*I, *Hpa*II, *Hin*fI, or *Hae*III) were purified by electroelution from 2% agarose gels, cleaved by the mutant endonucleases, and the sizes of the resulting fragments were determined by electrophoresis.

To determine the sites of scission at nucleotide resolution, uniquely ^{32}P-end-labeled f1 restriction fragments (Maniatis *et al.*, 1982) were subjected to either Maxam and Gilbert sequencing reactions (Eckert, 1987) or to cleavage by the wild-type or mutant enzymes. Approximately 0.5 ng (1000−2000 c.p.m.) of a ^{32}P-end-labeled restriction fragment and 10−20 ng of purified enzyme were incubated for 1 h at 30°C. The reactions were terminated at 65°C for 15 min, then sodium acetate (to 0.3 M) and carrier tRNA (10 μg) were added, and the reactions were divided and ethanol precipitated. One half was resuspended in 6 μl of 95% formamide, 0.1% xylene cyanol, and 0.3% bromophenol blue and displayed on 6% polyacrylamide−8M urea sequencing gels. The other half was resuspended in 10 μl of 10% sucrose, 25 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue and electrophoresed under non-denaturing conditions in 8−12% polyacrylamide gels containing 1 × TBE buffer (Maniatis *et al.*, 1982). The gels were dried onto Whatman 3 mm paper and exposed to Kodak XAR film at −70°C with intensifying screens.

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